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Tula Virus: a Newly Detected Hantavirus Carried by European Common Voles

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A novel hantavirus has been discovered in European common voles, *Microtus arvalis* and *Microtus rossiaemeridionalis*. According to sequencing data for the genomic RNA S segment and nucleocapsid protein and data obtained by immunoblotting with a panel of monoclonal antibodies, the virus, designated Tula virus, is a distinct novel member of the genus *Hantavirus*. Phylogenetic analyses of Tula virus indicate that it is most closely related to Prospect Hill, Puumala, and Muerto Canyon viruses. The results support the view that the evolution of hantaviruses follows that of their primary carriers. Comparison of strains circulating within a local rodent population revealed a genetic drift via accumulation of base substitutions and deletions or insertions. The Tula virus population from individual animals is represented by quasispecies, indicating the potential for rapid evolution of the agent.

Hantaviruses (genus *Hantavirus*, family *Bunyaviridae*) are agents carried by different rodent or insectivore hosts worldwide and exhibit a spectrum of pathogenicity for humans, ranging from inapparent and mild to highly lethal infections, which include hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome. Negative-stranded large (L), middle (M), and small (S) genomic RNA segments of about 6,500, 3,700, and 1,800 nucleotides (nt) in length encode the viral polymerase, two surface glycoproteins, G1 and G2 (processed from one precursor), and the nucleocapsid protein (N), respectively. Highly conserved 3′ and 5′ termini of the genomic RNA are complementary to each other, which results in the formation of panhandle structures (for a review see reference 13).

The three major pathogens, hantavirus serotypes Hantaan (HTN), Seoul (SEO), and Puumala (PUU), are the etiologic agents of the various forms of HFRS and are transmitted by contact with excretions of infected animals, i.e., striped field mice (*Apodemus agrarius*), urban and laboratory rats (*Rattus norvegicus* and *Rattus rattus*), and bank voles (*Clethrionomys glareolus*), respectively. The major clinical symptoms of HFRS are fever and acute renal failure with or without hemorrhagic manifestations. HTN is endemic in the Far East, causing HFRS with 3 to 7% mortality (25, 26). SEO is an urban pathogen (1 to 2% mortality) found worldwide (24, 26). PUU, found in Europe and Siberia, causes relatively mild HFRS (mortality, ~0.2%) (4, 26, 30). In the Balkan area, the yellow-necked field mouse (*Apodemus flavicollis*) carries a hantavirus called Dobrava (3) or Belgrade (18), reported as an etiologic agent of HFRS with high mortality (18). Hantavirus pulmonary syndrome is a newly detected deadly hantavirus-associated disease (76% mortality), first detected in May 1993 in the Four Corners area of the southwestern United States (12, 33). It is caused by a virus later named Muerto Canyon virus (MC) (6), transmitted to humans mainly from the deer mouse, *Peromyscus maniculatus*, a member of the *Sigmodontinae* subfamily of the *Muridae* family. According to sequencing data, MC is most closely related to Prospect Hill virus (PH) (33, 43). MC-related nucleotide sequences were found in deer mice trapped in 1983 in California (31), suggesting an archaic presence of the agent in the New World. A more recent report (6) indicates the existence of another, less pathogenic MC-related virus in the United States which is carried by the cotton rat, *Sigmodon hispidus*. Hantavirus serotypes PH (27), Thailand (14), and Thottapalayam (5), considered to be nonpathogenic to humans, are carried by meadow voles (*Microtus pennsylvanicus*), bandicoots (*Bandicota indica*), and shrews (*Suncus murinus*), respectively.

The accumulated data indicate that specific features of hantaviruses are determined primarily by their natural hosts (1, 39, 50). It should be noted that the presence of hantaviruses in many rodents species has not yet been studied. Thus, the possible existence of yet unknown hantaviruses in these hosts cannot be ruled out. The pathogenicity of these viruses to humans remains unpredictable because knowledge about the factors influencing virulence of hantaviruses is lacking.

Recently, we used PCR techniques to analyze wild hantaviruses and also to determine the sequences of the S segments of several wild PUU strains from natural HFRS foci in Finland and European Russia (36, 37). The experimental scheme developed was based on the use of genus-specific primers for the reverse transcription and PCR amplification of the full-length S segment which encodes the N protein, a major and highly conserved viral antigen. Additional features of the S segment such as having an evolutionary history similar to that of the M segment (37, 50) and insertion/deletion patterns at the 3′ noncoding region (NCR) make it a good model for studying microevolutionary events occurring at the genomic level (36). The 3′ NCR of the S segment is unusually long in

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hantaviruses, ~500 nt. It does not contain an open reading frame (ORF) for an additional protein such as those found in some members of the *Bunyaviridae* family, i.e., Phlebo, Uuku, and tomato spotted wilt viruses (13). Thus, an approach based on the sequencing of the S segment seemed appropriate for the analysis of genetic relationships of known hantaviruses as well as for studies of their evolution. With this approach, we have now discovered a new virus in lung samples of European common voles, which we call Tula virus (TUL).

MATERIALS AND METHODS

Rodents. European common voles, *Microtus arvalis* and *Microtus rossiaemeridionalis*, were trapped in 1987 in Tula, ~200 km south of Moscow, Russia. All voles belonged to the same local population. In enzyme immunoassay and immunofluorescence tests (8, 17), hantavirus antigen related to PUU and/or PH was found in seven rodents, six *M. arvalis* and one *M. rossiaemeridionalis*. These voles were selected for further investigation. Our attempts to isolate virus from the specimens by using Vero E6 cells have not been successful.

Reverse transcription-PCR cloning and sequencing of viral RNA from infected rodent tissues. Total RNA isolated from *Microtus* lung tissue was reverse transcribed in the presence of a genus-specific primer, 5′-TAGTAGTAGAC-3′. This was followed by amplification in PCR with the S-segment-specific primer 5′-TTCTGCAGTAGTAGTAGACTCCTTGAAAAG-3′ as described earlier (36, 37). The PCR products were cloned by using a SureClone ligation kit (Pharmacia Biotech) and sequenced by the dideoxy method, using Sequenase version 2.0 (United States Biochemical) according to the manufacturer's recommendations.

Recombinant TUL and PUU antigens. PUU and TUL recombinant N antigens were expressed as glutathione S-transferase (GST) fusion proteins with pGEX2T (Pharmacia) plasmids in *Escherichia coli* BL21(DE3). Expression was induced with 0.4 mM isopropyl-β-D-thiogalactoside. To prepare GST–PUU-$N_{\Delta 2}$, the PUU S region coding for the first 267 amino acids (aa) (47) was subcloned into pGEX2T. The resulting clone (pGEX-PUU-$N_{1\text{-}267}$) encoded a 63-kDa (63K) fusion protein. GST–PUU-$N_{\Delta 1}$, coding for the PUU N-terminal 118 aa, was prepared by amplification of the corresponding region from PUU S and subcloned into pGEX2T (22a). The resulting clone encoded a 40K fusion protein. To obtain GST–TUL-N, the entire coding region for the N protein was amplified by PCR from a cloned S segment of T76 with primers 5′-AGCTACGTCGACAAAGATCTGGAATGAG-3′ (initiation codon underlined) and 5′-GTGTCTGCAGGATCCGTT GATTAGATTTTTAGTGG-3′ (triplet complementary to the termination codon underlined). The amplified product was cut with *Bgl*II and *Bam*HI and cloned into the *Bam*HI site of pGEX2T. The resulting plasmid (pGEX2T-Tula-N) encoded a 76K fusion protein. GST–TUL-N_{Δ}, a GST fusion construct coding for the 61 N-terminal residues of TUL N protein, was prepared as follows. pGEX-Tula-N was cut with *Eco*RI and yielded the vector with the first 180 nt of the TUL N coding sequence. It was then ligated to form plasmid pGEX2T-Tula-$N_{1\text{-}61}$, encoding a 32K GST fusion protein.

MAbs. Bank vole monoclonal antibodies (MAbs) raised against PUU have been produced and characterized previously (29). The MAbs used in the present study have previously been shown to recognize seven different genus-, group-, or virus-specific epitopes in the PUU N protein. The reactivities of the MAbs with hantaviruses of different serotypes, determined by immunofluorescence tests, are listed in Table 2.

Immunoblotting assay. Recombinant and natural hantaviral N antigens were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% gel) and immunoblotted with bank vole MAbs to N protein, using cell culture supernatants at 1:100 dilutions. The incubations were carried out overnight at 4°C. The secondary antibody was a goat anti-mouse horseradish peroxidase conjugate (Dakopatts) used at a 1:400 dilution, and the incubations were carried out at 37°C for 1 h. The membranes were blocked, and antibodies were diluted in phosphate-buffered saline containing 1% nonfat dry milk. The washing buffer used was 50 mM Tris (pH 7.4)–5 mM EDTA–150 mM NaCl–0.05% Tween 20, and the bands were stained with *o*-phenylenediamine dihydrochloride.

Computer analyses. The program suite Genetic Data Environment (42) was used to manipulate the data and to calculate the least squares-additive trees (9) with Jukes-Cantor corrections. The neighbor-joining method (38) was implemented in the PHYLIP (16) package. The parsimony bootstrapping analysis (15) with PAUP (45) was done by using heuristic search, missing gap mode, random sequence addition, nearest-neighbor interchange branch swapping, and MULPARS options.

Nucleotide sequence accession numbers. The sequencing data for the full-size hantavirus S segment of four *M. arvalis* (no. 23, 53, 76, and 175) specimens and one *M. rossiaemeridionalis* (no. 249) specimen have been deposited in the EMBL Data Library with accession numbers Z30941 to Z30945. The corresponding virus strains, which were not isolated, were designated as follows (according to the nomenclature adopted [37]; abbreviated names are given in parentheses): TUL/23Ma/87 (T23), TUL/53Ma/87 (T53), TUL/76Ma/87 (T76), TUL/175Ma/87 (T175), and TUL/249Mr/87 (T249).

RESULTS

S-gene and N-protein sequences of TUL. Seven specimens containing hantavirus antigen related to PUU and/or PH, six from *M. arvalis* and one *M. rossiaemeridionalis*, were selected for an amplification test. All but one specimen were also found positive in reverse transcription-PCR, and the length of each of the amplified products was close to the expected 1,800 nt of the S segment. Finally, four full-size PCR products from *M. arvalis* (no. 23, 53, 76, and 175) and one from *M. rossiaemeridionalis* (no. 249) were cloned and sequenced. The sequencing data showed that the virus, designated TUL, is genetically related to but distinct from known members of the *Hantavirus* genus (Fig. 1).

The S segment of TUL (Fig. 1B) is 1,844 to 1,847 nt in length and contains in the messenger sense a 5′ NCR of 42 nt, an ORF of 430 aa, and a 3′ NCR of 509 to 512 nt, depending on the strain sequenced. There is an overlapping ORF2 (in the +1 position) with the coding capacity for 90-aa protein (~10K) resembling those in PUU and PH S segments. The distribution of nucleotide substitutions (Fig. 1A) shows that this region is one of the least variable in the S segment of TUL.

The organization of the S segment of TUL resembles that of other hantaviruses. However, while both the 5′ NCR and the coding regions of the TUL S segment could be aligned with the corresponding regions of other hantaviruses, a part of the 3′ NCR was unique for TUL (Fig. 1B). This part (nt 1370 to 1480) could not be reliably aligned with known hantaviral sequences. The region from nt 1480 to 1640 showed superficial similarity to the corresponding region of MC, but the significance of this is dubious because of the exceptionally high proportion of T residues.

Certain specific features of the TUL predicted N-protein sequence were revealed. Figure 1C depicts the part of the

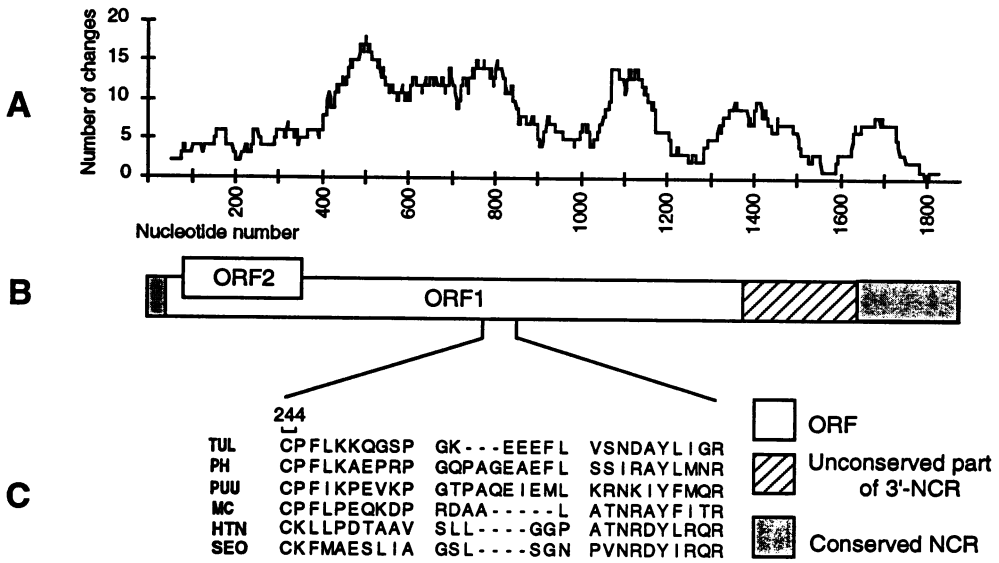

FIG. 1. Properties of the TUL S segment. (A) Distribution of nucleotide replacements in the S segment of five TUL strains from *M. arvalis* specimens 23, 53, 76, and 175 and from *M. rossiaemeridionalis* specimen 249 (T23, T53, T76, T175, and T249). The window size was 101. (B) Organization of the S segment plus-sense RNA. ORF1 and ORF2, ORFs for N protein and for a hypothetical nonstructural protein, respectively; Unconserved part of 3'-NCR, the part of the 3' NCR (nt 1370 to 1640) which was not reliable aligned to nucleotide sequences of other hantaviruses (for comments on the similarity of nt 1480 to 1640 to MC sequences, see text); Conserved NCR, the 5' NCR and the part of the 3' NCR which were aligned to sequences of other hantaviruses. (C) Alignment of N protein of hantaviruses (aa 244 to 273 in PUU). Data for MC (31a, 43), PH (35), PUU (the prototype Sotkamo strain) (47), HTN (40), and SEO (2) are from the indicated references. Dashes indicate deleted amino acids.

hantaviral N-protein sequence (aa 244 to 273) containing a highly variable region. In the area located between positions 254 and 263, three amino acids are deleted in TUL compared with PH and PUU. Four amino acids from the same region are deleted in HTN and SEO, and five are deleted in MC.

Phylogenetic analysis of TUL S-segment and N-protein sequences. In the least squares-additive phylogenetic tree (Fig. 2), TUL and PH form a separate branch of the genus. The monophyly of TUL and PH proved to be sensitive to the

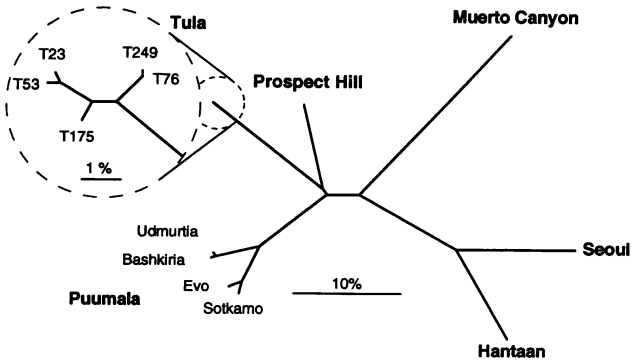

FIG. 2. Phylogenetic analysis of TUL S-segment sequences. The least squares-additive phylogenetic tree is based on hantaviral S-segment nucleotide sequences. Part of the sequence which was unique for TUL and corresponded to nt 1368 to 1647 in the PUU S segment as well as genus-specific terminal panhandle-forming nucleotide stretches were excluded from the analysis as uninformative. PUU strains: Sotkamo, prototype Sotkamo/V-2969/81 strain (47); Evo, Evo/12Cg/93 strain (36); Bashkiria, Bashkiria/Cg18-20/84 strain (44); Udmurtia, Udmurtia/894Cg/91 strain (37). Published sequences of PH (35), HTN (40), and SEO (2) S segments were used. The sequence of the MC S segment was kindly provided by S. Nichol.

starting values in distance-matrix methods. To take into account the observed transversion and transition disequilibrium in TUL (transition/transversion ratio; 4.5) and in PUU (ratio, 2.2), the parsimony analysis was applied with different weights. Any value in the range of 2 to 10 to favor transitions over transversions supported a separate TUL-PH branch in 72 to 92% of 100 bootstrap replicates. The mapping of TUL and PH within the same branch indicates the existence of an early shared ancestor for these two viruses. The sequence divergence between PH and TUL is comparable to that between HTN and SEO, two different serotypes, and about twofold higher than the divergence between different strains belonging to a single serotype (PUU). The S segments and N proteins originated from *M. arvalis* and *M. rossiaemeridionalis*, trapped at the same place and time, showed no pronounced differences, reflecting a close relationship between these two European common voles species (49) and indicating that TUL is probably carried by both sibling species.

The branch lengths and topology of trees based on N-protein amino acid sequences (not shown) supported the results from nucleotide data. TUL N protein (the sequence deduced from the strain T76 nucleotide sequence) shows higher relatedness to PH (81.8% of amino acids are identical), PUU (79.0 to 79.7%), and MC (73.4%) than to HTN (63.5%) and SEO (62.6%) N proteins.

Genetic variation of TUL within a local rodent population and in an individual rodent. Sequence analysis of five closely related TUL strains revealed genetic drift via nucleotide substitution, mostly transitions, and for the 3' NCR, deletion and insertions. Data on the frequency of mutations and the range of diversity within TUL strains are summarized in Table 1. For comparison, the corresponding values for PUU (36, 37), the only data available for other wild hantaviruses, are also given. As expected, the frequency of mutations as well as the range of diversity were higher for nucleotides than for amino

TABLE 1. Variation of TUL S gene and N protein within a local vole population and an individual vole (*M. arvalis*)

Level of analysis	Frequency of mutations (10^{-3})		Range of diversity (%)	
	S gene[a]	N protein	S gene	N protein
Vole population[b]	12.5 (4.8–6.7[c])	2.8 (2.3–2.9)	1.5–4.9 (0.3–2.0)	0.2–1.2 (0–1.2)
Individual vole[d]	0.9 (1.6–2.9)	2.0 (2.0)	0.2–0.6 (0.2–1.2)	0.6–1.7 (0.5–2.0)

[a] Coding region.
[b] Master sequences.
[c] Corresponding values calculated for PUU strains circulated in two geographically separated vole populations (36, 37).
[d] Fifty-five individual S-gene clones of strain T175 sequenced (nt 800 to 1350).

acids. Both sets of values calculated for the S-segment nucleotides were significantly higher for TUL than for PUU. However, at the protein level, these differences were not detected, indicating the presence of strong stabilizing selection.

S genes and N proteins of TUL from an individual vole were found to be a complex mixture of closely related variants, or quasispecies. Partial sequencing (nt 800 to 1350) of 55 individual clones originated from the S segment of a single *M. arvalis* specimen (no. 175) revealed, besides the master sequence, 27 altered ones. While clones with the master sequence represented about 50% of the viral population, each altered type was represented in the same small proportion, and all but one contained just a single nucleotide or/and amino acid alteration. In one case, the alteration resulted in the appearance of a termination codon at position 392 of the predicted protein sequence. One of the altered variants contained exactly the same substitution (nt 887, aa 282) which was found in the master sequence of the T249 strain. The existence of this particular variant within the T175 genome and the nonrandom distribution of mutations (data not shown) argue against an artificial (via PCR variations) nature of the mutant spectrum that we observed.

The frequency of amino acid substitutions determined for quasispecies was higher than that of nucleotide substitutions (Table 1). The range of diversity was also wider at the protein than at the RNA level.

Analysis of TULA with a panel of MAbs. TUL itself has not yet been isolated; therefore, naturally expressed antigen derived from the lung tissues of infected rodents and recombinant N antigen expressed as a GST fusion protein (see the legend to Fig. 2 for details) were examined by using a set of MAbs raised against PUU. The hantavirus genus-specific MAbs (1C12, 2E12, and 4E5) react with all hantavirus serotypes tested so far (reference 29 and Table 2). The group-specific MAbs (5B5 and 3G5) recognize more than one serotype, while virus-specific MAbs (3H9 and 5E1) react exclusively with PUU strains. The MAb analyses established that TUL N antigen (Table 2 and Fig. 3) was (i) a hantavirus-related antigen, (ii) differed from those of HTN, SEO, and PUU in the pattern of reactivity, and (iii) differed from PUU and PH N antigens in mobility in SDS-PAGE.

The genus-specific MAb 1C12 recognized the natural TUL N antigen (Fig. 3, lane 7) and showed no reactivity with the negative control sample from uninfected *M. arvalis* (lane 6). TUL N antigen is seen as a 48K band, as opposed to PUU and PH N antigens, which have the mobility of 50K (lanes 9 and 10).

The group-specific MAb 3G5 reacted strongly with both natural and recombinant PUU N antigens (Fig. 3, lanes 2, 3, and 9) but not with natural PH N or natural TUL N antigen in immunoblots (lanes 10 and 7). This antibody reacted very weakly with GST–TUL-N (lanes 4 and 5). The differences in 3G5 MAb reactivity with natural PH N antigen, strongly reactive in immunofluorescence assay but negative in immunoblotting, suggest that the corresponding epitope might be destroyed during the immunoblotting procedure. The PUU-specific MAb 5E1 reacted with both native and recombinant antigens exactly as MAb 3G5 did; i.e., it detected PUU N but not TUL N antigen (data not shown).

The N terminus of the protein was found to be highly antigenic; the fusion protein GST–TUL-N_{Δ}, containing the N-terminal 61 aa, reacted well with MAbs 1C12, 3G5 (Fig. 3), and 4E5 and with human nephropathia epidemica serum (data not shown). This result is in agreement with the reactivity of constructs expressing the N terminus of PUU N protein (Fig. 3, GST–PUU-$N_{\Delta 1}$) and loss of antigenicity of N-terminally-deleted PUU N proteins as reported earlier (20). The construct GST–PUU-$N_{\Delta 2}$, containing aa 1 to 267, has the epitopes of all MAbs used in this study (Table 2) and is thus comparable to GST–TUL-N.

DISCUSSION

TUL is a distinct novel members of the *Hantavirus* genus. The general organization of the S segment of TUL resembles that of other hantaviruses. Nevertheless, some specific features of both the nucleotide and predicted protein sequences are evident. The existence within the 3′ NCR of the S segment of a sequence unique for TUL suggests genetic divergence of TUL from other hantaviruses. This part of the S segment was preserved in a slightly variable form in all five TUL strains sequenced so far. The apparent random nature of the changes

TABLE 2. Reactivities of MAbs with hantavirus nucleoprotein epitopes

Antigen	Pattern of MAb reactivity in immunofluorescence[a]						
	N-a (3H9)	N-b (5E1)	N-c (5B5)	N-d (3G5)	N-f (1C12)	N-g (2E12)	N-h (4E5)
Natural							
PUU	+	+	+	+	+	+	+
PH	−	−	−	+	+	+	+
HTN	−	−	(+)	−	+	(+)	+
SEO	−	−	−	−	+	(+)	+
TUL[b]	−	−	−	−	+	+	+
Recombinant[c]							
GST–PUU-$N_{\Delta 2}$	+	+	+	+	+	+	+
GST–TUL-N	−	−	−	(+)	+	+	+

[a] See reference 29. Designations of epitopes are followed by MAb designations in parentheses. +, positive reaction; (+), weakly positive reaction; −, negative reaction.
[b] Lung tissues of *M. arvalis* (specimen 175) tested in immunoblotting.
[c] See the legend to Fig. 3 for details. GST–PUU-$N_{\Delta 2}$, containing aa 1 to 267, had the epitopes of all MAbs used in this study and is thus comparable to GST–TUL-N.

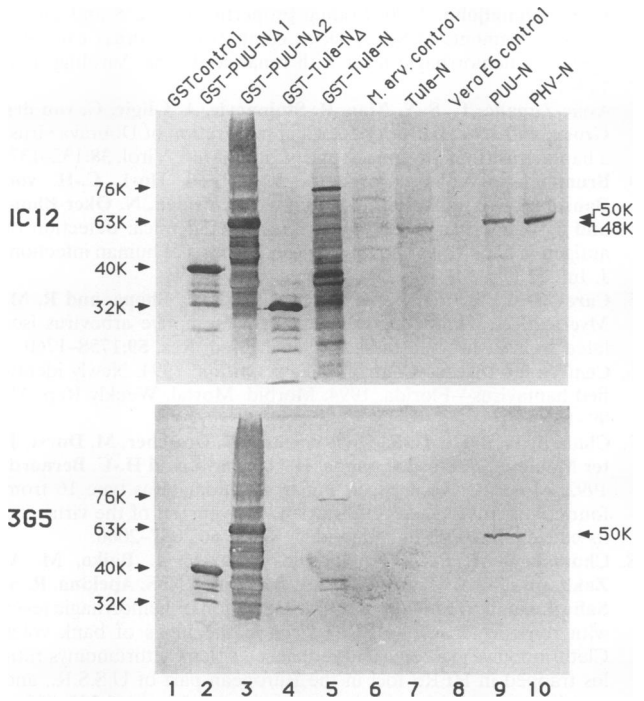

FIG. 3. Immunoblot analysis of TUL N antigen. Recombinant and natural hantaviral N antigens were immunoblotted with MAb 1C12 (top) and 3G5 (bottom). Lanes: 1, 22.5K GST; 2, GST–PUU-$N_{\Delta 1}$, a GST fusion of the 118 N-terminal residues of PUU Sotkamo strain N protein, 40K; 3, GST–PUU-$N_{\Delta 2}$, a GST fusion of the 267 N-terminal residues from PUU Sotkamo strain N protein, 63K; 4, GST–TUL-N_{Δ}, a GST fusion of the 61 N-terminal residues of TUL N protein, 32K; 5, GST–TUL-N, a GST fusion of TUL N protein's ORF, 76K; 6, *M. arvalis* control; 7, TUL N antigen; 8, Vero E6 control; 9, PUU N antigen; 10, PH N antigen. The *M. arvalis* control was a small (1- to 2-mm^3) piece of lung specimen from hantavirus-negative *M. arvalis* (trapped in Finland) that was stored in liquid nitrogen and for the immunoblot analysis homogenized and dissolved in 400 μl of Laemmli sample buffer, of which 10 μl was loaded on the gel. TUL N was a small piece of PCR-positive lung (TUL/175) which was prepared in the same manner as the *M. arvalis* control. The Vero E6 control was the total protein from 5×10^4 uninfected Vero E6 cells. PUU N antigen was the total protein from 5×10^4 Sotkamo strain-infected Vero E6 cells 14 days postinfection. PH N antigen was the total protein from 10^5 PH-1 strain-infected Vero E6 cells 7 days postinfection.

in this region and the lack of known related sequences suggest that it may not serve a specific function.

Phylogenetic analyses based on the aligned part of TUL S segment (Fig. 2) shows that TUL has diverged from other hantaviruses to the extent that it can be identified as a distinct genotype. The distance between TUL and PH was about four times longer than that between strains belonging to the same serotype. The sequence of the part of TUL N protein described in Fig. 1C was different from that in other hantaviruses. This area colocalizes with the region of highest S-segment/N-protein variability in hantaviruses (37, 46) and, moreover, with one of the major antigenic epitopes in PUU (28, 46). This particular region has been demonstrated to contain the target for one of the PUU-specific MAbs, 3H9 (28), which did not react with either natural or recombinant TUL N antigen (Table 3). Thus, this MAb might be useful for differentiation between PUU and TUL N antigens.

The immunochemical data obtained for TUL N antigen are in a good correlation with the results of sequencing. The reactivities of the genus-specific MAbs revealed the presence of three conserved epitopes and confirmed that the virus belongs to the *Hantavirus* genus. The recognition of recombinant TUL N by the group-specific MAb 3G5 was in line with the closer relationship to PUU and PH than to HTN and SEO. The lack of reactivity of MAbs 3H9 and 5E1 revealed that TUL N is antigenically distinct from PUU. Accordingly, sequencing demonstrated that the PUU-specific epitope of MAb 3H9 (aa 251 to 260) is missing in TUL N. Natural TUL N migrated as a 48K band in SDS-PAGE, as opposed to the 50K bands of PUU and PH. Since the lengths of the corresponding N proteins differ only by three to four amino acids, such significant difference has to be explained by other factors (e.g., alterations in secondary and tertiary structures). Similar mobility differences, i.e., to a higher extent than the predicted molecular weights, have previously been recorded between PUU and PH versus HTN and SEO N proteins (27a, 41).

Our analysis of the distribution of nucleotide substitutions within the region with potential double coding capacity, ORF2, revealed that this is one the least variable regions in the S segment of TUL (Fig. 1A) as well as of PUU viruses (our unpublished data), supporting the possibility that ORF2 is functional. Interestingly, a slightly shorter ORF2 was found in the S segment of MC (43) and the functional character of ORF2 was discussed on the basis of statistical analysis of the base substitution frequencies within this region in MC, PUU, and PH. ORF2 is found in all hantaviruses except HTN and SEO, which are carried by *Murinae* subfamily members and form a separate branch of the genus (Fig. 2). Also Bunyamwera, the prototype virus of the *Bunyaviridae* family, contains a similar ORF2 in about the same region of the S segment (13). Existence of the ORF2-encoded hypothetical nonstructural proteins awaits investigation.

When one compares the phylogenetic relationships among all members of the *Hantavirus* genus (Fig. 2) and their natural hosts (49), the evolution of hantaviruses seems to follow the evolution of their primary carriers. TUL and PH, carried by Old World and New World *Microtus* species, respectively, form a separate branch of the genus and thus seem to have originated from a shared early ancestor. Further, TUL and PH are mapped on the phylogenetic trees closer to PUU than to HTN and SEO. This reflects the fact that their natural hosts, *Microtus* and *Clethrionomys* species, both belong to the same subfamily, *Arvicolinae* (49). Finally, HTN and SEO, carried by the members of *Murinae* subfamily, are more closely related to each other than to other members of the genus. A working hypothesis explaining these facts might be as follows. An ancient virus was introduced into ancestors of modern rodent species and has been evolving together with them, diverging finally into the different genotypes and/or serotypes of current hantaviruses. The coevolution of viruses together with their hosts does not seem to be unique to hantaviruses; the same has been reported for human papillomaviruses (7).

Genetic variation in TULA: evidence for genetic drift and the potential for rapid evolution. Genetic variations in the S segment of TUL strains suggest the existence of genetic drift via accumulation of nucleotide substitutions and insertions or deletions. Data previously obtained for PUU strains (36) indicated that the evolutionary pattern of hantaviruses resembled that of vesicular stomatitis virus (32) rather than human influenza A virus (19); namely, there was a correlation to the geographical distribution of hantaviruses but not to the time of virus isolation. The mutation frequency of TUL strains (more than 10 substitutions in the S gene) is comparable to the values determined for other RNA viruses, e.g., for the foot-and-

mouth-disease virus strains isolated during a disease outbreak (60 to 100 substitutions per genome of ~8,500 nt) (10, 11, 22).

The host might very much determine the viral evolution (23). Thus, one of the possible reasons for the different nucleotide mutation frequencies in TUL and PUU is the difference between the host genera. First, the *Microtus* genus is evolutionarily younger than *Clethrionomys*; in particular, specially the *arvalis* group is still radiating (34). Second, because of the differences in social behavior, population oscillations are often more violent and hence densities both much higher and much lower in *Microtus* than in *Clethrionomys* species (21). Therefore, there are more severe "population bottlenecks" when only a few infected voles can survive. Later, with a rapid increase in the population size, a larger number of *Microtus* individuals are again susceptible to infection. Thus, founder effects (48) could promote the divergence of TUL.

Similar to the differences in virus strains, the genetic heterogeneity of TUL and PUU S quasispecies (Table 1) was comparable to that for other RNA viruses, e.g., for genomes of one foot-and-mouth-disease virus isolate (10, 11, 22). The detailed analyses of TUL quasispecies, including comparison of viral populations from several individual voles belonging to the same local population, will be published elsewhere.

Pathogenicity of TUL. There is not yet any answer to the question of whether TUL infects humans. One possibility is that the pathogenicity of this virus is similar to that of PH, which has not been known to induce any apparent illness in humans. Although antibodies reactive with PH were detected in mammologists (51), some doubts concerning the PH specificity of the immune response have arisen (43) because of the extensive serological cross-reactivity of PUU, PH, and related hantaviruses.

As mentioned above, our attempts to isolate TUL in Vero E6 cells have not been successful. It is well known that the adaptation of hantaviruses is tedious and the frequency of isolation is very low. The use of other cell lines would probably be more appropriate.

There is a high degree of cross-reactivity between TUL and PUU N antigens (our unpublished data); therefore, the various diagnostic assays currently in use for detection of PUU-specific antibodies would also recognize putative TUL-reactive antibodies in human sera. Consequently, if TUL infects humans with a pathogenicity similar to that of PUU, such infections could be serologically incorrectly diagnosed as PUU infections. We are at the present developing TUL-specific assays, based on recombinant proteins and MAbs, and have recently started regular screening of human sera collected in the Tula region. Preliminary data showed that several HFRS patients from this region had higher immunoglobulin G endpoint titers to recombinant TUL N antigen than to recombinant PUU N antigen.

Rapid accumulation of data concerning new and emergent hantaviruses some of which might be highly pathogenic suggests that more attention should be given to surveillance of the agents in different rodent and insectivore populations.

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